JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2010, p. 957–959 Vol. 48, No. 3
0095-1137/10/$12.00 doi:10.1128/JCM.01678-09

Cytospin-Enhanced Immunofluorescence and Impact of Sample Quality on Detection of Novel Swine Origin (H1N1) Influenza Virus[▽]

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Received 28 August 2009/Returned for modification 28 October 2009/Accepted 19 December 2009

Nasopharyngeal swabs ($n = 601$) from 278 adult and 323 pediatric patients were tested within 24 h of receipt by cytospin-enhanced direct immunofluorescence antibody testing (DFA) and real-time reverse transcriptase PCR (RT-PCR) using the CDC assay. Cytospin-enhanced DFA detected 230 (84.6%) of 272 swine influenza A PCR-positive results overall but 25 (92.6%) of 27 positive results in patients less than 5 years old and 208 (96.7%) of 215 positive samples with cycle threshold values of <26.

The advent of swine origin influenza virus (S-OIV) or novel H1N1 virus in the late spring of 2009 raised concerns about the sensitivity of diagnostic tests to detect this virus (2). To date, two studies have been published on direct immunofluorescence antibody testing (DFA) with very different results (3, 7). The Clinical Virology Laboratory at Yale New Haven Hospital has performed cytospin-enhanced DFA for 10 years and has reported an overall sensitivity for seasonal influenza of over 95% compared to cell culture (4–6). In this paper, we present our experience with cytospin-DFA in detecting S-OIV, or novel H1N1, compared to the Centers for Disease Control and Prevention (CDC) real-time PCR protocol.

Over an 11-week period in May to July of 2009, 601 nasopharyngeal (NP) swabs were tested within 24 h of receipt by both cytospin-enhanced DFA (SimulFluor respiratory screen reagents; Millipore Inc., Temecula, CA) and TaqMan PCR. One swab was collected per patient and placed in M4 medium (Remel, Lenexa, KS). After centrifugation to pellet cells and resuspension in a small amount of phosphate-buffered saline (PBS), a 200-μl aliquot of concentrated cell suspension was applied to single-well slides using a cytospin and then fixed and stained as previously described (4). PCR was performed using the CDC real-time reverse transcriptase PCR (RT-PCR) protocol for influenza (www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html) using 45 cycles of amplification on an ABI 7500 instrument (Foster City, CA). Samples were screened by pan A RT-PCR, and if positive, tested for seasonal H1 and H3, swine A, and swine H1 by RT-PCR. Patient ages ranged from 7 weeks to 87 years, and the study included 278 adult patients and 323 pediatric patients. Only 48 patients (8.0%) were less than 5 years old.

Positive results by pan A, swine A, and swine H1 influenza RT-PCR confirmed S-OIV. Samples positive by pan A and swine PCR but negative by subtype PCR for H1, H3, and swine H1 were presumptive S-OIV. PCR-positive samples by pan A PCR ($n = 7$) only, or by seasonal H1 ($n = 3$) or H3 ($n = 9$) PCR, were excluded from analysis. Twenty-five samples with fewer than 20 respiratory epithelial cells were deemed inadequate for DFA and were excluded. One of these 25 samples was positive for S-OIV with cycle threshold (C_T) values of 35 by swine A PCR and 37 by swine H1 PCR.

The results for the remaining samples are shown in Table 1. Cytospin-DFA detected 230 (84.6%) of 272 swine A PCR-positive samples, as well as 20 non-influenza virus infections (17 parainfluenza infections, 2 respiratory syncytial virus [RSV] infections, and 1 adenovirus infection). The specificity of DFA was 99.3%. When the data were analyzed by the age of the patient (Table 2), DFA detected 78 of 93 (83.8%) S-OIV PCR-positive samples in patients 18 years of age and older, 127 of 152 (83.5%) in patients 5 to 17 years of age, and 25 (92.6%) of 27 in patients <5 years old. DFA results correlated with the C_T values (Table 2). For samples with swine A C_T values of <26 cycles, 208 of 215 (96.7%) were DFA positive. For PCR-positive samples with C_T values of ≥26 cycles, only 22 of 57 (38.6%) were DFA positive. For S-OIV-positive samples, swine A PCR had C_T values on average 1 cycle lower than pan A PCR and 1 to 4 cycles lower than swine H1 PCR. According to the CDC influenza PCR protocol, a strong positive PCR was defined as C_T value of <30 cycles. Twenty samples were low positive with $C_T \geq 30$ cycles by both pan A and swine A PCR, and 9 of these had C_T values of >34 cycles. Eight of 9 with C_T values of >34 cycles were swine H1 PCR negative. None of these 8 swine H1 PCR-negative samples were DFA positive.

Two papers with DFA results for novel H1N1 have been reported, with widely different assessments of DFA sensitivity (46.7 versus 93%). In a high-volume hospital referral laboratory, 2,861 samples from patients of all ages were tested by DFA using D3 respiratory virus reagents (3). Samples, including NP flocked swabs, which are purported to improve sample quality (1), were collected from a variety of local hospitals and clinics. The criteria for sample adequacy for DFA were not given. The overall sensitivity of DFA was 46.7%. In the same report, BinaxNOW detected only 9.6%

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▽ Published ahead of print on 30 December 2009.

TABLE 1. Comparison of cytospin-enhanced DFA and real-time TaqMan RT-PCR for swine origin influenza virus (S-OIV)

Swine influenza A virus RT-PCR result	No. of samples with the following cytospin-DFA result for influenza A virus:			Total no. of samples
	Positive	Negative	Indeterminate[a]	
Positive	230	33	9	272
Negative	2[b]	275[c]	8	285
Total no. of samples	232	310	17	557

[a] Atypical staining requiring confirmation by PCR or culture.
[b] Two hospitalized children, one 12-year-old child with pneumonia and one 9-month-old child with bronchiolitis, had 1 or 2 influenza virus-positive cells by DFA.
[c] SimulFluor cytospin-DFA also detected 17 parainfluenza infections, 2 RSV infections, and 1 adenovirus infection. The sensitivity, specificity, positive predictive value, and negative predictive value for cytospin-DFA were 84.6%, 99.3%, 99.1%, and 87.2%, respectively.

of novel H1N1-positive samples. The poor results may reflect the poor quality of samples received in reference laboratories, despite the use of flocked swabs. It is also possible that the extremely high sample volume experienced in this laboratory may have overwhelmed the capacity of the laboratory to perform manual tests and contributed to a decline in performance.

In contrast, Pollock et al. tested only 112 samples over an 11-day period and deemed 42 PCR positive (7). Trained hospital respiratory therapists collected two flocked swabs per patient to enhance specimen quality. At least 30 columnar epithelial cells per test well were required for an adequate sample, a higher standard than the 20 cells commonly used. For inclusion as novel H1N1 PCR positive, all 3 PCR assays (pan A, swine A, and swine H1) had to be positive at the State Laboratory. From experience in our own laboratory, these criteria could have eliminated some PCR low-positive samples. Despite testing samples from adults who shed lower titers of virus, DFA had an excellent sensitivity of 93% compared to PCR. The significantly enhanced sample collection protocol, together with the possible exclusion of low positive PCR samples, likely contributed to the high DFA sensitivity.

In our study, 601 samples were obtained by a variety of collectors who were not specially trained. Although flocked swabs were not used, cytocentrifugation enhanced the number of cells per slide. Our DFA detection rate of 84.6% and our Binax detection rate of 38% (data not shown) were significantly higher than the 46.7% and 9.6% values, respectively, reported by Ginocchio et al. (3). These differences could be due to better sample quality, as well as methodological differences. As expected, a positive DFA result correlated with PCR C_T value, which is a reflection of viral load. Compared to Pollock et al., our criteria for a positive PCR allowed the inclusion of more low-positive samples, which were more likely to be DFA negative. Inclusion of these 8 low-positive samples (pan A and swine A PCR positive, swine H1 and seasonal H1, H3 PCR-negative samples) reduced our DFA sensitivity from 87.1% (230/264) to 84.6% (230/272). If the 7 samples positive only by pan A PCR (C_T values of 32 to 40) were included as likely S-OIV positives, the sensitivity of DFA would be further reduced to 82.4%. Though not proven to be S-OIV, these repeatable low-positive pan A PCR results were reported to clinicians as influenza A positive in the peak of the outbreak. Thus, to exclude them from the analysis may inappropriately elevate the sensitivity of the DFA. Of note, compared to seasonal influenza, the pediatric patients tested for novel H1N1 influenza virus were older, which may have contributed to lower viral titers in the samples.

In conclusion, the performance of DFA for novel H1N1 influenza virus varies in different settings and is highly dependent on sample quality as well as technical expertise. DFA can be performed in routine virology laboratories, with results available in 2 h, and can also detect multiple viral pathogens if desired. More effort should be devoted to improving sample quality through trained collectors, collection of two swabs in one vial, and the use of flocked swabs and cytospin-prepared slides. Excellent results can be obtained even in adults. Although PCR is the definitive clinical test for novel H1N1, especially for high-volume reference laboratory testing, DFA performed on-site in the hospital laboratory with rapid turnaround time can make an important contribution to S-OIV diagnosis and patient management. Clinicians should be advised that a concerted effort to im-

TABLE 2. Effects of patient age and viral load on DFA sensitivity for S-OIV[a]

Range of swine A influenza virus PCR C_T values[b]	No. of samples in range (n = 272)	No. of cytospin-DFA-positive samples/no. of samples tested (%) by patient age and C_T value		
		<5 yrs	5–17 yrs	≥18 yrs
12–19	113	13/13 (100)	58/58 (100)	42/42 (100)
20–25	102	10/10 (100)	56/61 (91.8)	29/31 (93.6)
26–30	43	2/3 (66.7)	11/25 (44.0)	6/15 (40.0)
31–35	9	0/1 (0)	2/6 (33.3)	1/2 (50.0)
36–40	5	0	0/2	0/3 (0)
Total no. of positive samples/total no. of samples tested (%)[c]	230/272 (84.6)	25/27 (92.6)	127/152 (83.5)	78/93 (83.8)

[a] Abbreviations: S-OIV, swine origin influenza virus; C_T, cycle threshold, the amplification cycle at which the real-time PCR result crosses the threshold to positive (the lower the C_T, the higher the viral load present in the sample).
[b] The PCR C_T value is a reflection of viral load. Seven samples positive by pan A PCR only were excluded; all were DFA negative. Eight samples positive by pan A and swine A PCR but negative by swine H1 PCR were included; 7 were DFA negative and one was DFA indeterminate.
[c] Of samples with swine A PCR C_T of <26, 208/215 (96.7%) were DFA positive. Of samples with a C_T of ≥26, 22/57 (38.6%) were DFA positive.

prove sample quality substantially benefits all test methods, but particularly nonamplified tests.

The expertise of Robin Garner and Sandra Cohen and the entire Clinical Virology Laboratory staff in performing the testing and of Gerri Russo in collecting the data are greatly appreciated.

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